

Coordinately Expressed in the Mouse Uterus with the Initiation of Implantation and Decidualization

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Two-way interactions between the blastocyst trophoblast and the uterine luminal epithelium are essential for implantation. The key events of this process are cell–cell contact of trophoblast cells with uterine luminal epithelial cells, controlled invasion of trophoblast cells through the luminal epithelium and the basement membrane, transformation of uterine stromal cells surrounding the blastocyst into decidual cells, and protection of the “semiallogenic” embryo from the mother’s immunological responses. Because cell–cell contact between the trophoblast epithelium and the luminal epithelium is essential for implantation, we investigated the expression of zonula occludens-1 (ZO-1) and E-cadherin, two molecules associated with epithelial cell junctions, in the mouse uterus during the periimplantation period. Preimplantation uterine epithelial cells express both ZO-1 and E-cadherin. With the initiation and progression of implantation, ZO-1 and E-cadherin are expressed in stromal cells of the primary decidual zone (PDZ). As trophoblast invasion progresses, these two molecules are expressed in stroma in advance of the invading trophoblast cells. These results suggest that expression of these adherence and tight junctions molecules in the PDZ serves to function as a permeability barrier to regulate access of immunologically competent maternal cells and/or molecules to the embryo and provide homotypic guidance of trophoblast cells in the endometrium. © 1999 Academic Press

Key Words: ZO-1; E-cadherin; implantation; uterus; embryo; mouse.

INTRODUCTION

The establishment of pregnancy results from successful blastocyst implantation in the uterus. Unlike tissue transplants, the uterus is considered an immunologically privileged site because the “semiallogenic” embryo, despite its genetic incompatibility, is not rejected during pregnancy by the immunologically competent mother (Billingham, 1964). This suggests that perhaps the trophoblast fails to express histocompatibility antigens or is simply not antigenic (Simmon and Russels, 1962). However, histocompatibility antigens are shown to be expressed in the trophoblast, implying that normal embryonic development *in utero* is protected

by the surrounding maternal decidua (Kirby *et al.*, 1966). It is speculated that the embryo is guarded by a physical (anatomical) barrier which allows the mother to develop a state of transplantation immunity against foreign histocompatibility antigens expressed in the trophoblast at the maternal–embryonic interface (Kirby *et al.*, 1964). However, the molecular nature of this barrier is not fully understood.

The process of implantation involves a series of cell–cell communications between the two epithelial tissues, trophoblast and luminal epithelium. Prior to implantation, both of them are two separate entities and are polarized with a continuous seal of junctional complexes and cell adhesion molecules. With the onset of the attachment reaction, trophoblast and luminal epithelial cells make

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first contact at their apical borders, at which point the trophoblast cells become invasive (Cross *et al.*, 1994; Strickland and Richards, 1992; Thie and Denker, 1997). For this interaction, both the receptive luminal epithelium and the invading trophoblast should modify their functional programming via changes in cell-surface molecules. In the mouse, the invasion of trophoblast cells through the luminal epithelium alters polarity and junctional complexes. This invasive nature of hemochorial placentation mimics that of highly invasive tumors, giving the normal trophoblast a pseudomalignant status (Strickland and Richards, 1992). Thus, the uterus must precisely limit trophoblast invasion during normal pregnancy. For example, decidual derived α_2 -macroglobin, a potent inhibitor of metalloproteinase, and tissue inhibitors of metalloproteinases are considered to limit trophoblast invasion (Gu *et al.*, 1992; Das *et al.*, 1997). However, the mechanisms by which this is achieved are not fully known.

The uterine luminal epithelial cells, like other epithelial cells, are united by symmetric "adherence junctions." In most cases, these junctions are underlaid with a dense 10- to 30-nm-thick cytoplasmic submembranous plaque to which bundles of cytoskeletal filaments attach. Two major and widespread plaque-bearing junctions, the adherence junctions and the desmosomes, usually coexist in the subapical regions of juxtaposed cells (Ishikawa *et al.*, 1981; Tsukita and Tsukita, 1989). The classical cadherins (E-cadherin, N-cadherin, and P-cadherin) are a family of Ca^{2+} -dependent cell-cell adhesion molecules which are highly concentrated at the adherence junctions. Each cadherin shows strong homophilic binding activity, which is important for cell sorting and guidance (Nose *et al.*, 1988). The tight junctions (zonulae occludentes) are components of the epithelial junctional complexes. These junctions seal the epithelial cells at their apices to create either a primary barrier or a gate for diffusion of solutes via the paracellular pathway. Tight junctions also function as a fence which maintains epithelial cell polarization by regulating the apical and basolateral polar distribution of plasma membrane proteins (Citi, 1993; Gumbiner, 1993).

One of the tight junction proteins is zonula occludens-1 (ZO-1), with a molecular mass of 220 kDa (Itoh *et al.*, 1997). In tight junctions of epithelial and endothelial cells, ZO-1 is localized in the cytoplasmic surface of plasma membranes (Anderson *et al.*, 1988; Stevenson *et al.*, 1988). There is also evidence that ZO-1 and cadherins are colocalized in cells lacking tight junctions (Itoh *et al.*, 1991, 1993). Cingulin (140 kDa) is localized to the region of the tight junction and

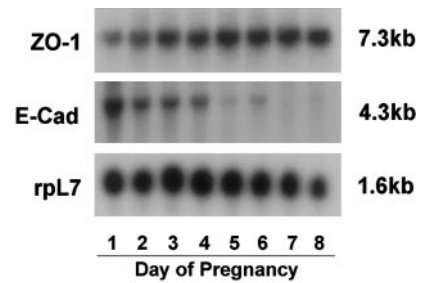


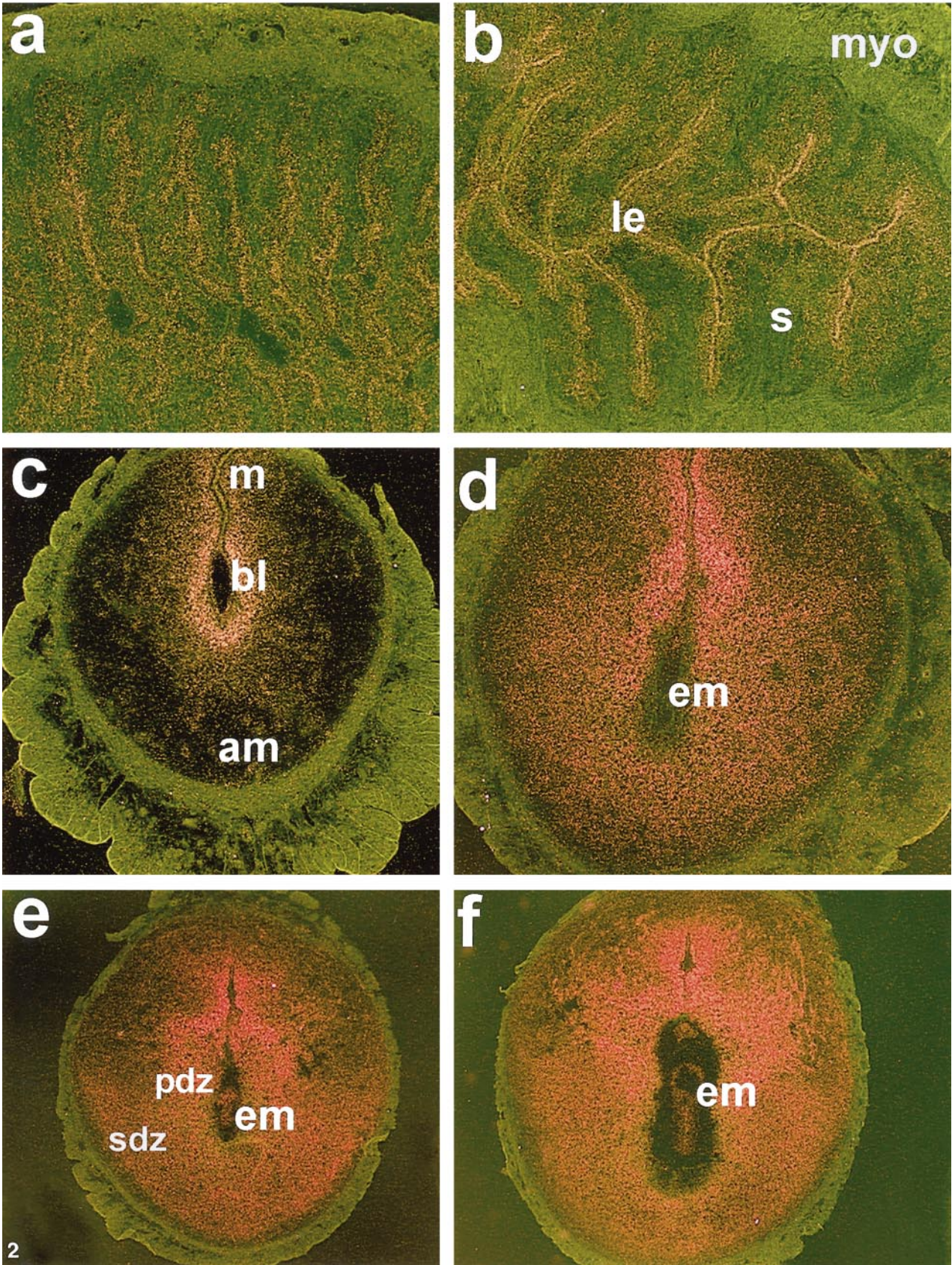
FIG. 1. Northern blot analysis of ZO-1 and E-cadherin mRNAs in the periimplantation mouse uterus. The mRNA levels were detected in poly(A)⁺ RNA samples obtained from whole mouse uteri from days 1–8 of pregnancy. A 7.3-kb transcript for ZO-1 mRNA and a 4.3-kb transcript for E-cadherin (E-Cad) were detected in the uterus. The blot was hybridized with rpL7 to confirm integrity, equal loading, and blotting of RNA samples.

is not tightly associated with the membrane (Citi *et al.*, 1988, 1989). Occludin (65 kDa) is localized at tight junctions and is directly associated with ZO-1 (Ando-Akatsuka *et al.*, 1996; Furuse *et al.*, 1993, 1994; Gumbiner, 1993).

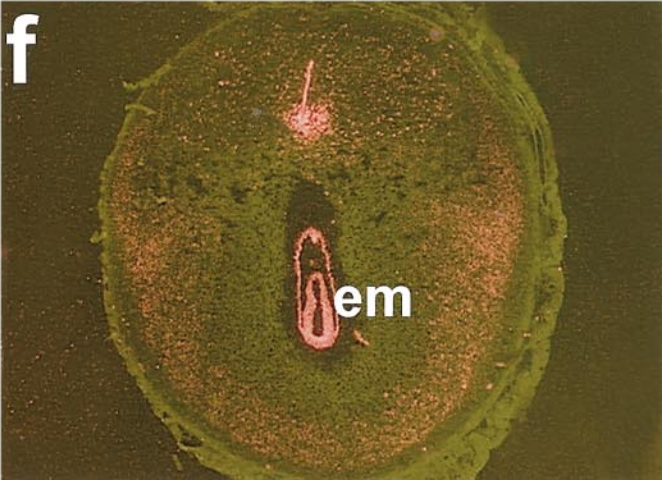
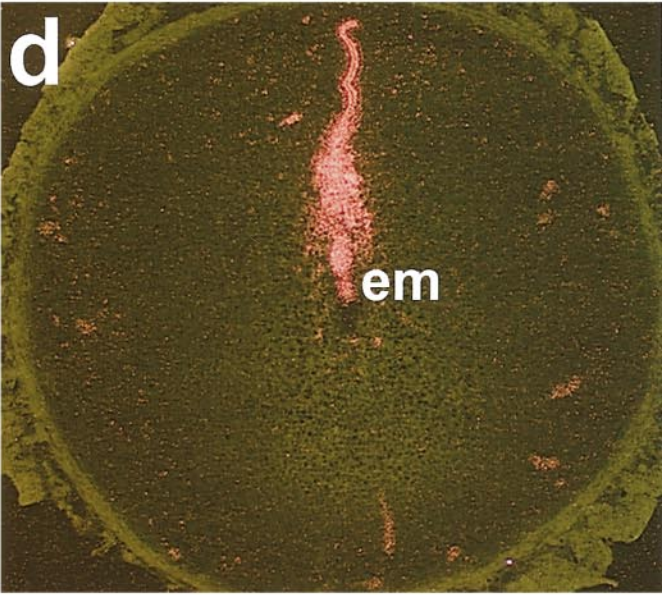
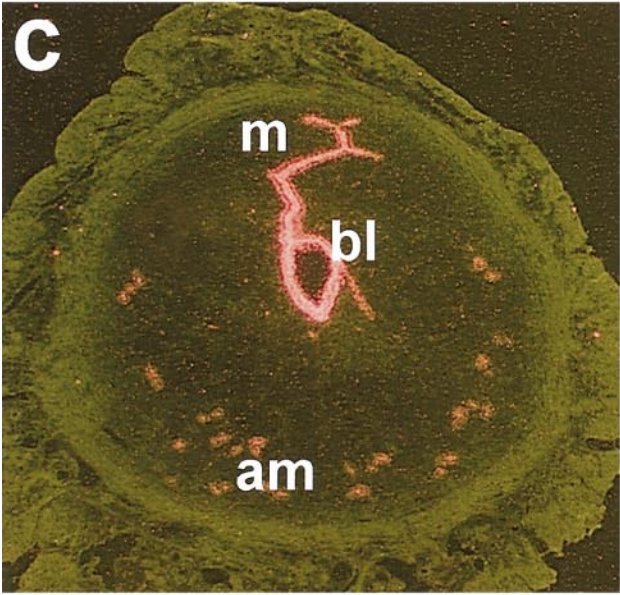
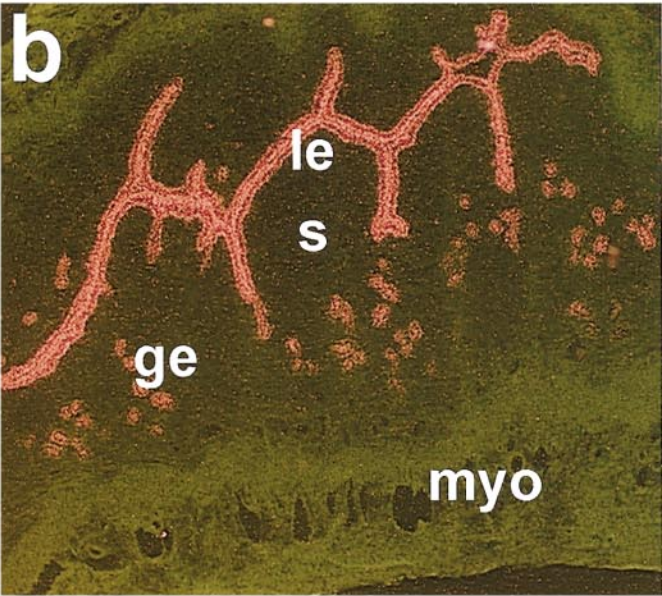
Around the time of the blastocyst attachment reaction in mice (Das *et al.*, 1994), the luminal epithelium remains closely apposed to the blastocyst trophectoderm and cell-cell contact is established between these two cell types. Luminal epithelial cells surrounding the embryo undergo apoptosis and facilitate penetration (Parr and Parr, 1986a,b). From day 5 onward, trophectoderm cells invade the luminal epithelium and reach the basal lamina at the antimesometrial pole (El-Shershaby and Hinchliffe, 1975; Enders and Schlafke, 1967; Finn and Hinchliffe, 1964). The adjacent decidual cells in fact first penetrate the epithelial basal lamina. The sloughing of epithelial cells from the basement membrane at the implantation chamber is observed after the formation of the primary decidual zone (PDZ) (Enders and Schlafke, 1967). The mesenchymal cells immediately surrounding the implanting blastocyst transform into decidual cells, first forming the cup-shaped PDZ (Krehbiel, 1937). The PDZ is composed of three to five tightly packed cell layers. This avascular zone is considered to function as a partial permeability barrier between the implanting blastocyst and the maternal circulation (Enders and Schlafke, 1967; Rogers *et al.*, 1983). While molecules smaller than 45 kDa (e.g., horseradish peroxidase) are freely permeable,

FIG. 2. *In situ* hybridization of ZO-1 mRNA in the periimplantation mouse uterus. Dark-field photomicrographs of representative uterine sections on day 1 (a), day 4 (b), day 5 (c), day 6 (d), day 7 (e), and day 8 (f) of pregnancy are shown with a–d at 40× and e–f at 20× original magnifications. am, antimesometrial side; m, mesometrial side; bl, blastocyst; em, embryo; le, luminal epithelium; s, stroma; myo, myometrium; pdz, primary decidual zone; sdz, secondary decidual zone.

ZO-1



E-cadherin



those larger than 45 kDa (e.g., immunoglobulins, microorganisms, and immunocompetent cells) are impermeable. Ultrastructural studies show that tight junctions present in the PDZ are incomplete (Parr and Parr, 1986a,b). The molecular nature of this permeability barrier and its physiological significance are not clearly understood. Our present findings demonstrate that ZO-1 and E-cadherin are expressed in the uterus predominantly at the sites of blastocyst implantation. The results suggest that the presence of these junctional proteins at the implantation sites surrounding the invading trophoblast creates a barrier that is conducive to embryonic development and implantation.

MATERIALS AND METHODS

Reagents. Rat monoclonal antibody against E-cadherin was a generous gift from Dr. R. Kemler, Max-Planck Institut für Immunobiologie (Freiburg, Germany). Rat monoclonal antibody against ZO-1 was purchased from Chemical International, Inc. (Temecula, CA). Biotinylated goat anti-rat secondary antibody and Histostain-Plus immunoreagents were purchased from Zymed Laboratories (San Francisco, CA). Other reagents were obtained from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Animals and treatments. All experiments with animals were conducted in accordance with NIH standards for the care and use of experimental animals. Virgin CD-1 female mice (48–60 days of age, 20–25 g; Charles River Laboratories, NC) were mated with fertile or vasectomized males of the same strain to induce pregnancy or pseudopregnancy, respectively. The morning of finding a vaginal plug was designated day 1 of pregnancy or pseudopregnancy. Mice on days 1–4 were killed at 0830–0930 h, and embryos were recovered from the reproductive tract to confirm pregnancy. On days 5–8, mice were killed at 0900 h. Implantation sites on day 4 (2300 h) and on days 5 and 6 were visualized by intravenous injection (0.1 ml/mouse) of a Chicago blue dye solution (0.1% in saline). Implantation sites were demarcated by discrete blue bands along the uterus (Paria et al., 1993). On days 7–8 (0900 h), implantation sites are distinct and blue dye injection is not required. Uterine tissues were flash-frozen for Northern hybridization, *in situ* hybridization, and immunocytochemistry.

Hybridization probes. A full-length mouse cDNA encoding ZO-1 inserted in pBluescript SK at *EcoRI* sites was kindly provided by Drs. M. Itoh and Sh. Tsukita, Kyoto University (Sakyo-ku, Kyoto, Japan). A small fragment (1.44 kb) was released with *AccI* and subcloned into a pGEM3ZF(+) vector. A 590-bp mouse cDNA inserted in Puc 8 at an *EcoRI* site was generously provided by Dr. R. Kemler, Max-Planck Institut für Immunobiologie. This was subcloned in the pGEM7ZF(+) vector. For Northern hybridization, the

antisense ^{32}P -labeled cRNA probes were generated using SP6 polymerase. For *in situ* hybridization, sense and antisense ^{35}S -labeled cRNA probes were generated using T7 and SP6 polymerases, respectively. A part of the ribosomal protein L-7 (rpL7) cDNA (246 bp, bases 359–604) was subcloned into a PCR-Script vector containing a promoter for T7 polymerase and used as a template for synthesis of a ^{32}P -labeled antisense rpL7 probe (Lim and Dey, 1997). All probes had specific activities of about 2×10^9 dpm/ μg .

Northern blot hybridization. Total RNA was extracted from whole uteri by a modified guanidine thiocyanate procedure (Das et al., 1994; Han et al., 1987). Poly(A)⁺ RNA was isolated by oligo(dT)-cellulose column chromatography (Sambrook et al., 1989). Poly(A)⁺ RNA (2 μg) was denatured, separated by formaldehyde-agarose gel electrophoresis, transferred, and cross-linked to the membrane by UV irradiation (Spectrolinker; Spectronics Corp., Westbury, NY). Northern blots were prehybridized, hybridized, and washed as described previously (Das et al., 1994, 1995). The same blots were sequentially hybridized to ZO-1, E-cadherin, and rpL7 probes, and the hybrids were detected by autoradiography.

In situ hybridization. *In situ* hybridization was performed as described previously (Das et al., 1994, 1995). On specific days of pregnancy, uterine horns were excised and cut into small pieces or separated into implantation and interimplantation sites. Frozen sections (10 μm) were mounted onto poly-L-lysine-coated slides. When required, frozen sections were cut serially to detect the sites of blastocysts. Sections were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at 4°C. Following prehybridization, uterine sections were hybridized to ^{35}S -labeled ZO-1 and E-cadherin sense or antisense cRNA probes for 4 h at 45°C. After hybridization and washing, the slides were incubated with RNase A (20 $\mu\text{g}/\text{ml}$) at 37°C for 15 min. RNase A-resistant hybrids were detected after 1–3 days of autoradiography using Kodak NTB-2 liquid emulsion. The slides were poststained with hematoxylin and eosin. Reddish brown grains represent autoradiographic signals.

Immunohistochemistry. Immunohistochemistry was performed as described previously (Das et al., 1994, 1995; Lim et al., 1997; Paria et al., 1998). In brief, frozen uterine sections (10 μm) were mounted onto poly-L-lysine-coated glass slides and fixed in cold acetone for 10 min followed by three washes in PBS (10 min each). Rat monoclonal ZO-1 or E-cadherin antibodies were used for immunostaining using a Zymed Histostain-Plus Kit (Zymed Laboratories). After immunostaining, sections were lightly counterstained with hematoxylin. Red deposits indicated the sites of immunoreactive proteins.

Immunoprecipitation and immunoblotting. Day 6 implantation sites (200 mg) from two mice were homogenized and extracted in chilled buffer (1% Triton, 1% NP-40, 1 mM EDTA, 1 mM PMSF, 1 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ pepstatin in 50 mM Tris, pH 7.4). The extract was centrifuged and the supernatant was incubated either with 10 μg of antibodies to E-cadherin or ZO-1 conjugated with protein A-Sepharose. The beads were washed with the same buffer, and the bound proteins were eluted by boiling the beads in SDS-PAGE sample buffer for 5 min. After centrifugation at 10,000

FIG. 3. *In situ* hybridization of E-cadherin mRNA in the periimplantation mouse uterus. Dark-field photomicrographs of representative uterine sections on day 1 (a), day 4 (b), day 5 (c), day 6 (d), day 7 (e), and day 8 (f) of pregnancy are shown with a–d at 40 \times and e–f at 20 \times magnifications. am, antimesometrial side; m, mesometrial side; bl, blastocyst; em, embryo; ge, glandular epithelium; le, luminal epithelium; s, stroma; myo, myometrium; pdz, primary decidua zone; sdz, secondary decidua zone.

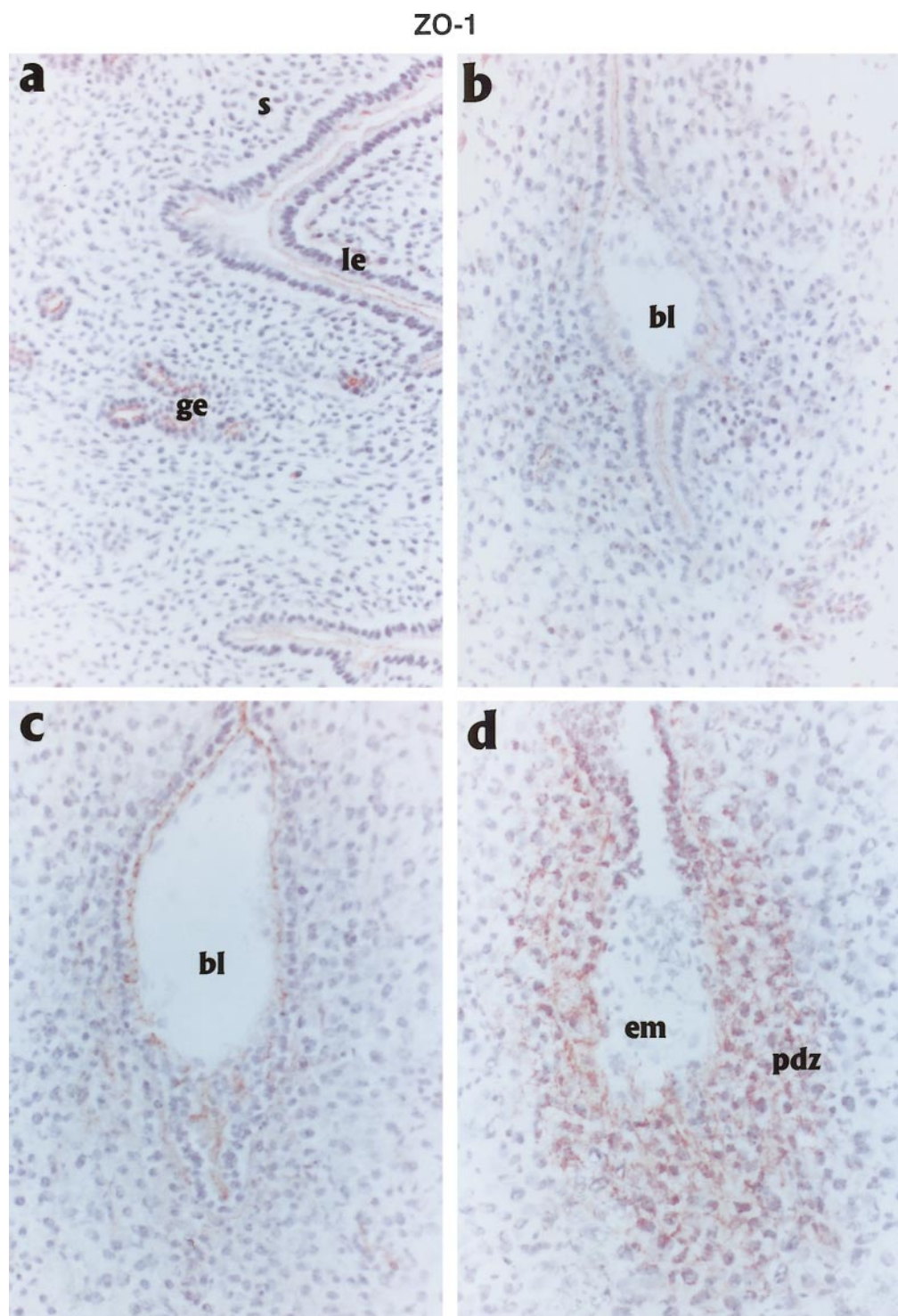
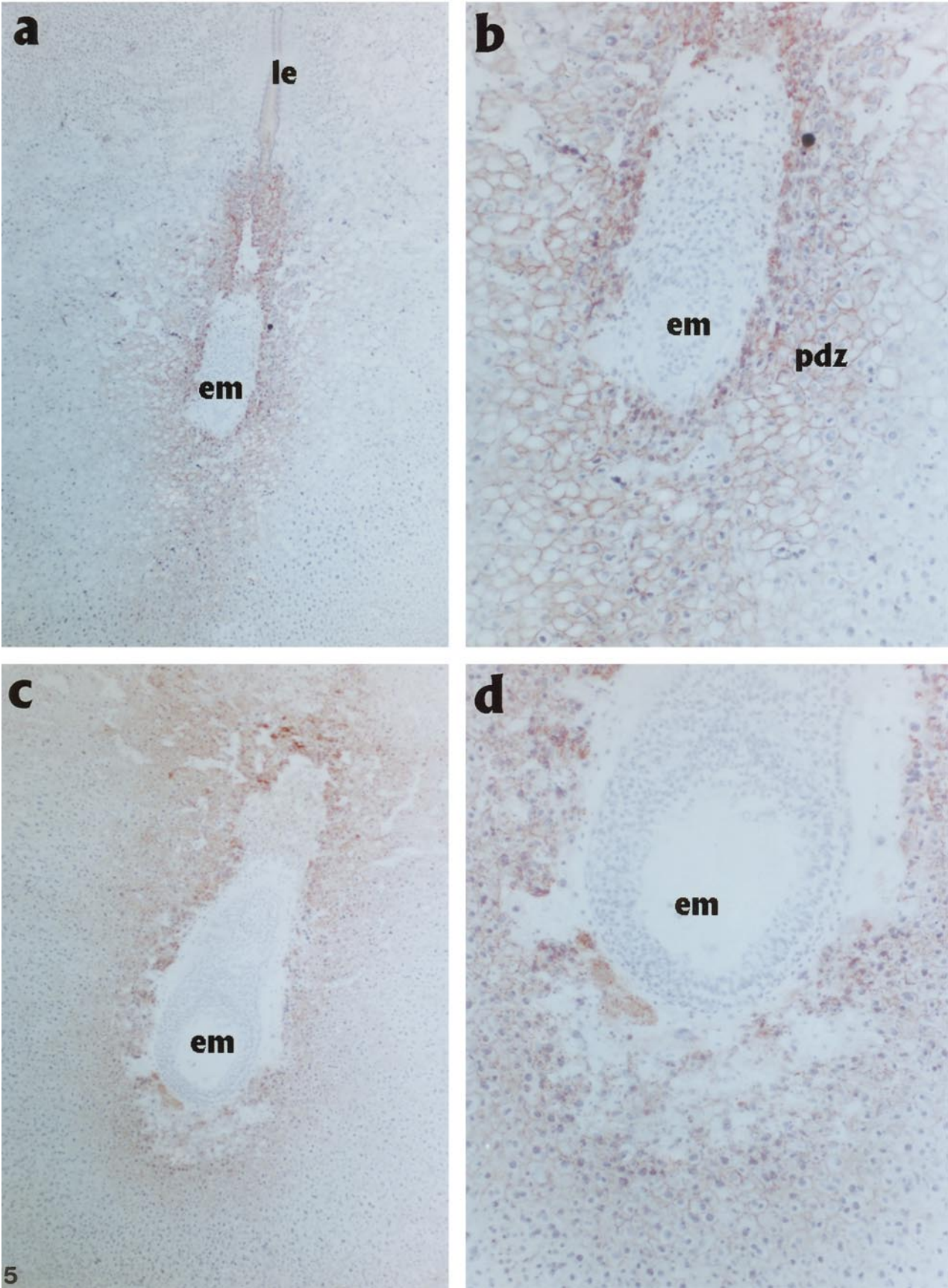


FIG. 4. Immunocytochemistry of ZO-1 in the mouse uterus on days 4–6 of pregnancy. Bright-field photomicrographs of representative uterine sections on day 4 at 0900 h (a), day 4 at 2300 h (b), day 5 at 0900 h (c), and day 6 at 0900 h (d) are shown at 40 \times magnification. bl, blastocyst; em, embryo; ge, glandular epithelium; le, luminal epithelium; s, stroma; pdz, primary decidua zone.

ZO-1



rpm for 5 min, the supernatant was collected and loaded (80 μ g of protein in each lane) onto 7.5% polyacrylamide minigels. Proteins were transferred onto Immobilon membranes which were subsequently blocked in 20% nonfat dry Carnation milk in TBS and incubated overnight with the primary antibodies (Paria *et al.*, 1994, 1998). After washes, membranes were probed with a 1:5000 dilution of secondary antibodies coupled to horseradish peroxidase for 1 h at 25°C and developed with enhanced chemiluminescence reagents (Amersham International, Buckinghamshire, England).

RESULTS

Northern blot analysis of ZO-1 and E-cadherin mRNAs in the periimplantation mouse uterus. Steady-state levels of whole uterine ZO-1 and E-cadherin mRNAs on days 1–8 of pregnancy were examined by Northern blot hybridization using 32 P-labeled cRNA probes. As in other tissues (Jesaitis and Goodenough, 1994; Itoh *et al.*, 1993), a 7.3-kb transcript of ZO-1 mRNA was detected in uterine RNA samples (Fig. 1). Levels of uterine ZO-1 mRNA remained virtually unaltered during the periimplantation period. In contrast, levels of E-cadherin mRNA (4.3 kb) (Ringwald *et al.*, 1987) were highest on day 1 of pregnancy followed by a marked decline from day 5 to 8. The integrity and loading of RNA samples were examined by rehybridizing the same blot to a rpl 7 probe (Fig. 1).

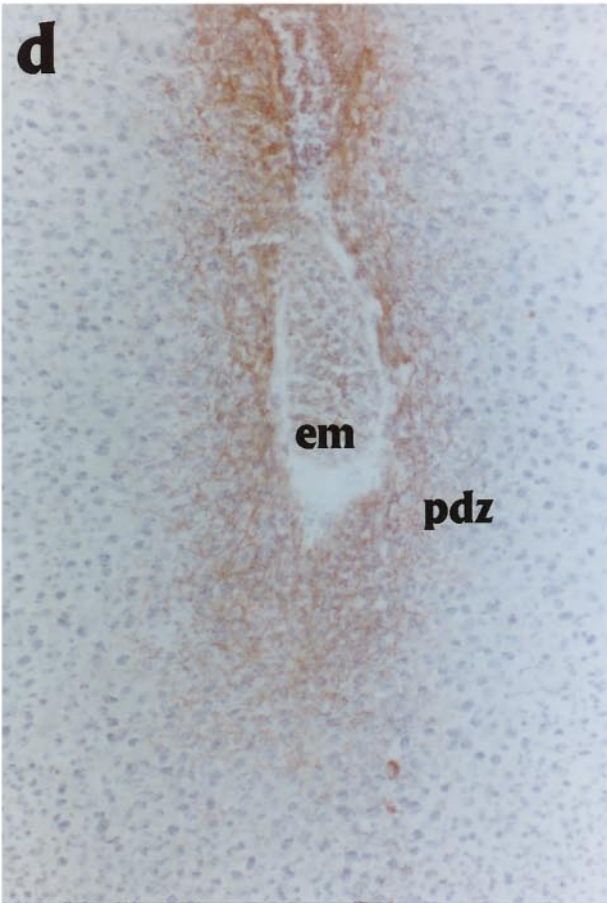
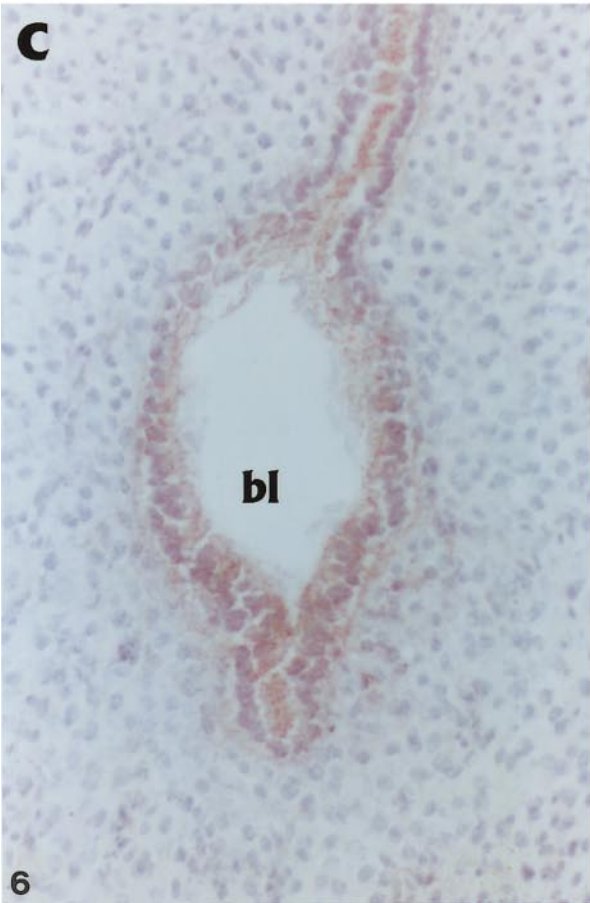
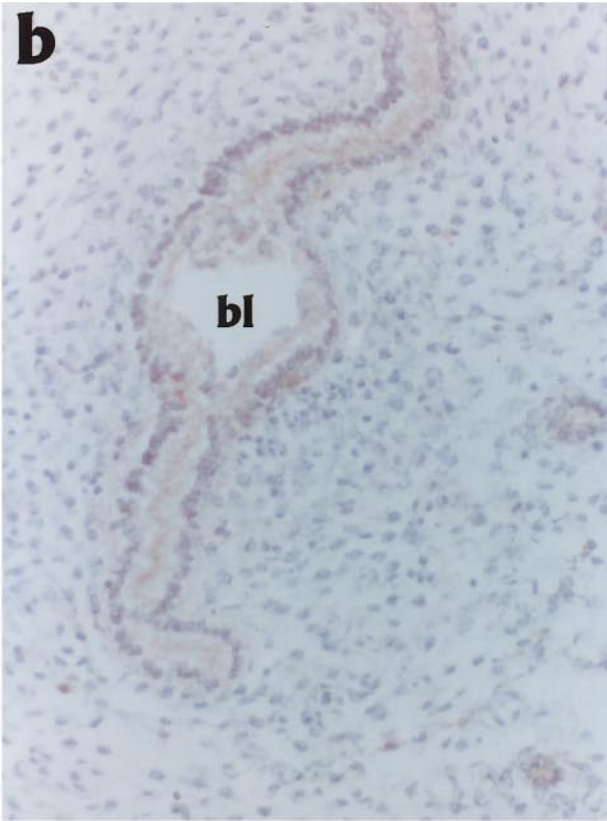
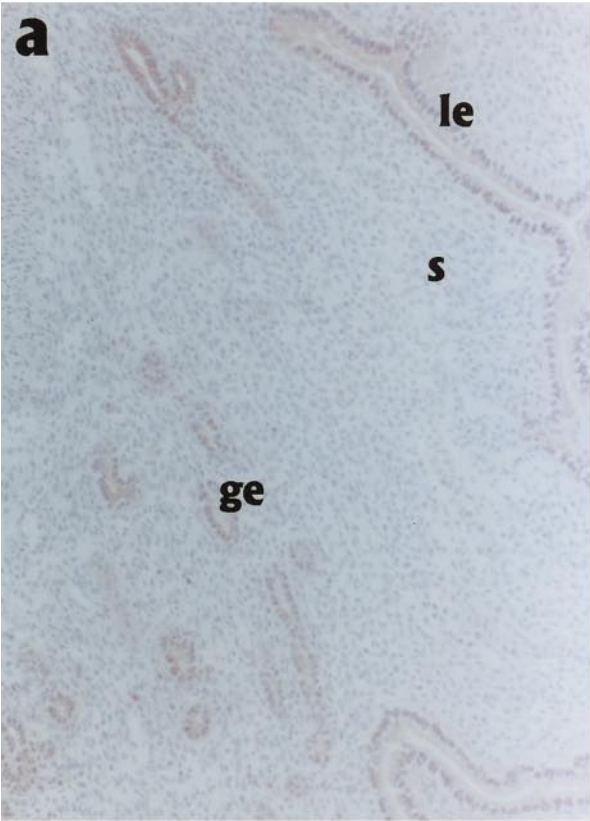
Cell-specific expression of ZO-1 and E-cadherin mRNAs in the periimplantation uterus by *in situ* hybridization. On days 1–4 of pregnancy, ZO-1 mRNA accumulated in uterine epithelial cells and in subepithelial stromal cells [Figs. 2a (day 1) and 2b (day 4)]. On day 5 of pregnancy, ZO-1 mRNA accumulation was primarily restricted to epithelial and subepithelial stromal cells surrounding the blastocyst at the site of implantation (Fig. 2c). On day 6 (Fig. 2d), the accumulation persisted into decidualizing stromal cells surrounding the implanting blastocyst (PDZ). Low levels of accumulation were also detected in cells outside the PDZ, the secondary decidual zone (SDZ). On days 7 and 8 (Figs. 2e and 2f), stromal cells in the SDZ and cells close to luminal epithelium at the mesometrial pole showed considerable accumulation of ZO-1 mRNA. Levels of accumulation were little lower in stromal cells at the antimesometrial pole. On days 6–8, the embryo also accumulated ZO-1 mRNA. Localization of this mRNA in the epithelial and stromal cells throughout the periimplantation period is consistent with unaltered levels of mRNA during this period as determined by Northern hybridization. E-cadherin mRNA was localized mainly in luminal and glandular epithelial cells on days 1–4 of pregnancy [Figs. 3a (day 1) and 3b (day

4)]. On day 5, this mRNA showed distinct accumulation in the luminal epithelial cells of the implantation chamber with little accumulation in surrounding stromal cells (Fig. 3c). On day 6 (Fig. 3d), accumulation was observed mainly in the surviving luminal epithelium at the mesometrial side of the implantation chamber and in underlying stromal cells. Low levels of E-cadherin mRNA were also detected in stromal cells of the PDZ. On days 7–8 (Figs. 3e and 3f), this mRNA accumulated primarily in the luminal epithelium, in the subepithelial stroma at the mesometrial side, and, at low levels, in the SDZ at the antimesometrial side. Implanting embryos on days 6–8 also expressed this mRNA. Sections hybridized with sense probes for ZO-1 or E-cadherin did not exhibit any positive signals (data not shown). The decline in the steady-state levels of E-cadherin mRNA on days 5–8 as determined by Northern hybridization is reflective of rapidly expanding decidua that represent bulk of the uterine tissue, but contributes little to the E-cadherin mRNA pool.

Cell-specific accumulation of ZO-1 and E-cadherin proteins in the periimplantation uterus. ZO-1 is expressed in luminal and glandular epithelial cells in the morning of day 4 of pregnancy (Fig. 4a). At the time of the blastocyst attachment to the luminal epithelium on day 4 at 2300 h, ZO-1 is localized in a continuous line at the interface between the trophectoderm and the luminal epithelium (Fig. 4b). Outside the area of direct blastocyst contact, apical ZO-1 expression of the luminal epithelia appeared to run in parallel, suggesting that the lumen is closed by tight junctions between epithelial cells. Perinuclear translocation of ZO-1 was not apparent in the epithelium, and no accumulation of ZO-1 was evident in stromal cells. Similarly, E-cadherin is localized in luminal and glandular epithelial cells on the morning of day 4 (0900 h) (Fig. 6a). On day 4 at midnight, the locale of the luminal closure at the site of implantation appeared to be sealed with E-cadherin. At this time, E-cadherin was seemingly localized at the apical surface of the luminal epithelium and in the lateral aspect of some luminal epithelial cells (Fig. 6b). On day 5, ZO-1 appeared to be localized mostly at the apical side with extension into the lateral borders of intact epithelial cells of the luminal epithelium, giving the arching shape appearance of the apically localized ZO-1 (Fig. 4c). However, high-resolution localization using immunogold labeling and electron microscopy will be required to ascertain more precisely the subcellular locale of the proteins. A modest amount of ZO-1 was present in the stroma underneath the luminal epithelium at the antimesometrial side of the implantation chamber. The localization of E-cadherin in

FIG. 5. Immunocytochemistry of ZO-1 in mouse uterus on days 7 and 8 of pregnancy. Bright-field photomicrographs of representative uterine sections on day 7 at 0900 h (a, 40 \times and b, 100 \times) and day 8 at 0900 h (c, 40 \times and d, 100 \times) are shown. em, embryo; le, luminal epithelium; pdz, primary decidual zone.

E-cadherin



the luminal epithelium apparently was more intense at this time (Fig. 6c). This protein was also present in subepithelial stromal cells around the implantation chamber.

On day 6 of pregnancy, ZO-1 (Fig. 4d) was localized predominantly in the PDZ, although it still persisted on the luminal surface of the epithelium outside the areas which had direct contact with the blastocyst. At this stage, luminal epithelial cells remaining at the site of implantation undergo apoptosis. Although E-cadherin was also localized in the PDZ on day 6 (Fig. 6d), it showed a differential localization pattern. E-cadherin was more abundantly localized in the luminal epithelium and subepithelial stromal cells at the mesometrial pole of the implantation chamber. Consistent with previous observations (Vestweber *et al.*, 1987; Nose and Takeichi, 1986), E-cadherin was also present in the developing embryo.

On day 7 of pregnancy, ZO-1 was localized in the PDZ at the antimesometrial pole and in the stroma at the mesometrial pole (Figs. 5a and 5b). ZO-1 was also present in the remaining intact luminal epithelium at the mesometrial side (Figs. 5a and 5b). On this day, E-cadherin was localized in the PDZ but was rather limited within the three to five layers of decidual cells. This protein was more prominently localized in stromal cells immediately above the implanting embryo at the mesometrial pole (Figs. 7a and 7b). The decidual cells distant from the embryo and at the antimesometrial pole had relatively less E-cadherin. On day 8, ZO-1 was localized mainly in the PDZ (Figs. 5c and d). It appears that it is not the decidualization process which induces ZO-1 expression in the decidual cells, rather the contact or close location of decidual cells with trophoblast cells initiates the production of ZO-1. On this day, E-cadherin persisted in the decidual tissue surrounding the developing embryo (Figs. 7c and 7d).

Although localization of immunoreactive E-cadherin mostly matches with that of the mRNA, the accumulation of protein in a larger area especially on day 7 may reflect faster turnover of the mRNA than that of the protein. The localization of ZO-1 protein is restricted to the epithelium prior to implantation and in the epithelium and PDZ after the initiation of implantation. In contrast, accumulation of ZO-1 mRNA is widespread, i.e., in the epithelium and stroma before implantation and in the epithelium, PDZ, and SDZ after implantation. This pattern may result from either rapid turnover of ZO-1 protein or inefficient translation of the mRNA in the stroma and SDZ. However, sensitivity of the two different assay systems to localize protein and mRNA cannot be directly correlated and should be interpreted with caution.

Association of ZO-1 with E-cadherin. To determine whether ZO-1 is tightly associated with E-cadherin complex, immunoprecipitation of an extract prepared from day 6 implantation sites was performed with both E-cadherin and ZO-1 antibodies. Both ZO-1 (220 kDa) and E-cadherin (120 kDa) were identified in these complexes. Further, immunoprecipitation with anti-E-cadherin antibodies verified the presence of ZO-1 (Fig. 8A), and immunoprecipitation with anti-ZO-1 antibodies confirmed the presence of E-cadherin (Fig. 8B). These combined results suggest that E-cadherin and ZO-1 are closely associated.

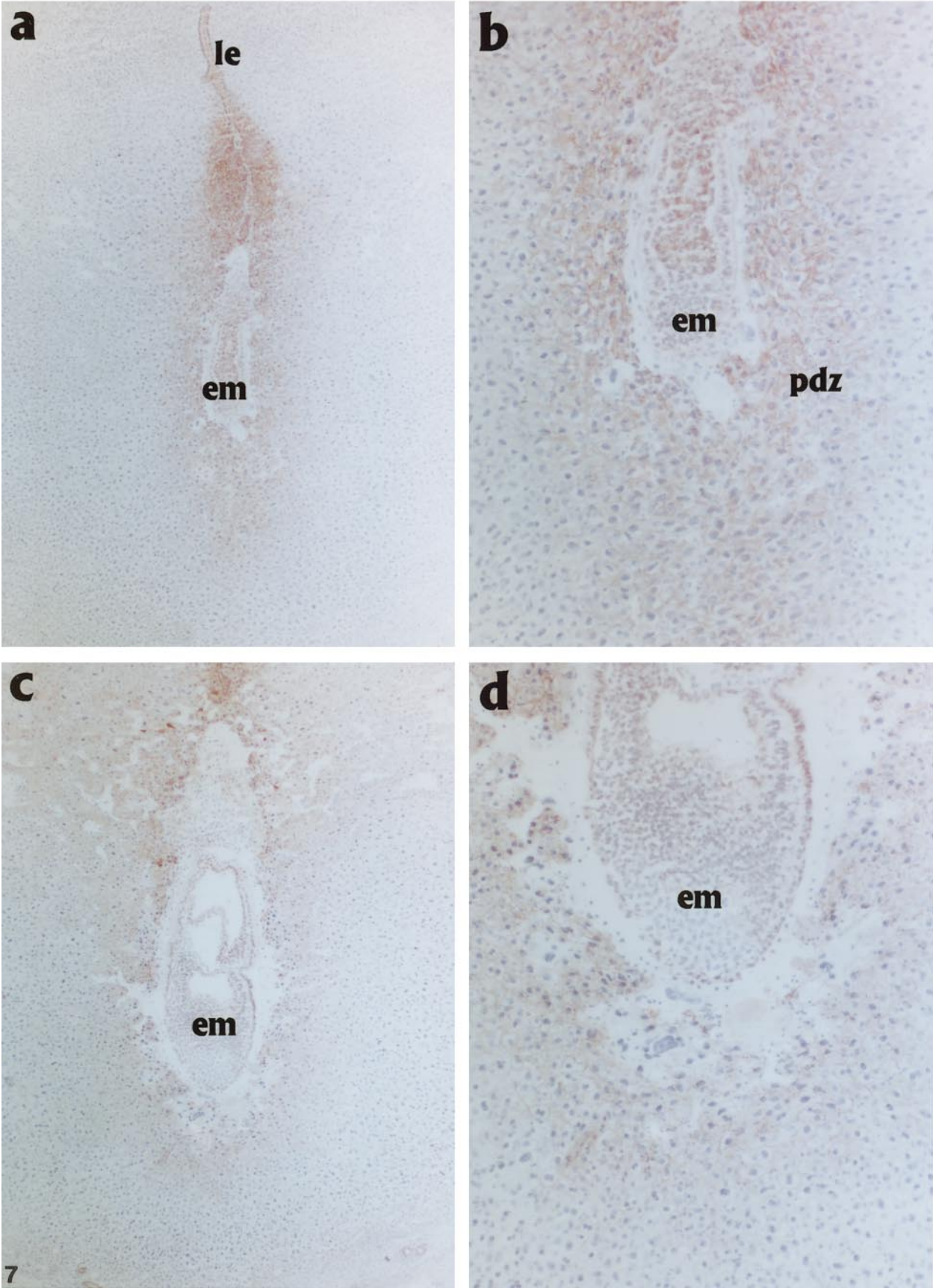
DISCUSSION

Both the trophectoderm and the luminal epithelium are independent entities and interactions between these two epithelia mark the beginning of implantation. The present investigation studied the behavior of the epithelial junctions and their two major components (ZO-1 and E-cadherin) as the two epithelial tissues attach, interact, and participate in interactive invasion. Prior to the attachment of the blastocyst to the luminal epithelium, the uterus becomes receptive for blastocyst implantation. The mouse uterus becomes receptive on day 4 of pregnancy (Paria *et al.*, 1993). As a marker of the receptive endometrium, we sought to identify epithelial changes with respect to the distribution of ZO-1 and E-cadherin, since ZO-1 distribution is altered or localized in the nucleus under hormonal and other stimuli (Singer *et al.*, 1994; Gottardi *et al.*, 1996). Our present results show that with initiation of trophoblast invasion through the luminal epithelium on day 5, ZO-1 appears to be localized to the apical and lateral aspects of the epithelium with perinuclear localization in some epithelial cells. This may suggest the beginning of loss of epithelial cell polarity. Similar localization pattern of ZO-1 was not noted in the morning or evening of day 4, eliminating ZO-1 as a suitable marker of uterine receptivity.

In the mouse, the acquisition of invasive properties by trophoblast cells, first at the antimesometrial and later at the mesometrial poles, constitutes an essential step in the progression of implantation. The trophoblast cells permeate the luminal epithelium to anchor the implanting embryo into the decidualizing stromal bed, a process achieved by a series of membrane-mediated events occurring between embryonic and maternal cells. Both the trophectoderm and the luminal epithelial cells are composed of a continuous monolayer of cells joined by various types of junctional

FIG. 6. Immunocytochemistry of E-cadherin in the mouse uterus on days 4–6 of pregnancy. Bright-field photomicrographs of representative uterine sections on day 4 at 0900 h (a), day 4 at 2300 h (b), day 5 at 0900 h (c), and day 6 at 0900 h (d) are shown at 40× magnification. bl, blastocyst; em, embryo; ge, glandular epithelium; le, luminal epithelium; s, stroma; pdz, primary decidual zone.

E-cadherin



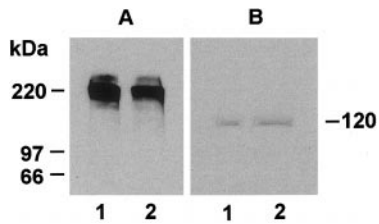


FIG. 8. Coimmunoprecipitation analysis of uterine ZO-1 and E-cadherin proteins. Coimmunoprecipitation was carried out as described under Materials and Methods. (A) Immunoblotted with ZO-1 antibody; (B) immunoblotted with E-cadherin antibody. Lane 1, immunoprecipitated with ZO-1 antibody; lane 2, immunoprecipitated with E-cadherin antibody. Molecular weights of ZO-1 (220 kDa) and E-cadherin (120 kDa) are indicated.

structures and adhesive molecules. These structures and molecules create a permeability barrier, without which free exchange of substances through the paracellular pathway will occur. Although many classes of cell adhesion molecules have been identified, the adhesiveness itself is principally governed by differential cadherin expression. Cadherins are Ca^{2+} -dependent cell-cell adhesion molecules which are specifically expressed in the epithelium and participate in the maintenance of epithelial phenotype, homotypic interactions, and cell guidance (Armstrong, 1989; Drubin and Nelson, 1996; Geiger and Ayalon, 1992). Adhesion via cadherins involves the coordination of extracellular binding and intracellular anchorage to the actin-based cytoskeleton. E-cadherin seems to be a regulator of the differentiated noninvasive epithelial cell state (Hordijk *et al.*, 1997).

We observed E-cadherin expression in luminal epithelial cells prior to implantation. With trophoblast invasion through the luminal epithelial cell basement membrane into decidualizing stromal cells after the onset of implantation, both the trophoblast and the cells at the PDZ express E-cadherin. This suggests that E-cadherin-positive stromal cells behave like epithelial cells to homotypically guide the trophoblast invasion into the stroma. The switching of invasion from the antimesometrial pole to the mesometrial pole with the progression of implantation is correlated with similar shifting of E-cadherin expression. Thus, the initial phase of implantation could be viewed as a process in which a pool of stromal cells behaves like epithelial cells at the antimesometrial pole of the implantation chamber. This mesenchymal-epithelial transformation may be required

for successful uterine anchorage of the embryo. The invasive nature of trophoblast cells at the implantation site appears to be different from that of cells involved in tumor invasion. In general, E-cadherin downregulation is correlated with malignancy parameters such as tumor progression, loss of differentiation, invasion, and poor prognosis (Bracke *et al.*, 1996). However, E-cadherin was also found to be expressed homogeneously in invasive tumors (Kadowaki *et al.*, 1994; Matsuura *et al.*, 1992). This suggests that E-cadherin alone may not be sufficiently active as an invasion-suppressive molecule. The implantation process is one in which E-cadherin and adherence junctions may play roles in cell remodeling by guiding and limiting trophoblast cell migration into the endometrium.

Epithelial cells also express tight junctions with different phenotypes and biological roles (Farquhar and Palade, 1963; Gumbiner, 1987; Stevenson *et al.*, 1988). The adherence junctions are required for tight junction organization and maintenance (Gumbiner, 1987). ZO-1 is expressed in both trophoblast cells and uterine epithelial cells prior to implantation, in the PDZ cells following implantation. The presence of ZO-1 in nonepithelial cells has previously been reported (Howarth *et al.*, 1992; Itoh *et al.*, 1993), and it is expressed before tight junctions appear in nonneuronal tissues during development (Colins and Flaming, 1995; Fleming and Hay, 1991). In cells with "less developed" tight junctions, ZO-1 localizes to both the tight and the adherence junctions (Itoh *et al.*, 1993), suggesting ZO-1's role in both types of junctions. In nonepithelial cells, ZO-1 is primarily concentrated at cell-cell adhesion sites where the cadherins are associated with actin-based cytoskeleton (Itoh *et al.*, 1991, 1993). The coimmunoprecipitation of ZO-1 with E-cadherin in extracts of day 6 implantation sites is consistent with this observation.

Electron microscopy and freeze-fracture studies revealed incomplete tight junctions in the PDZ (Tung *et al.*, 1986). These incomplete tight junctions were shown to function as semipermeable barriers to transport of large molecules through the paracellular pathway of the PDZ cells to the embryo. The avascular PDZ creates a potentially anoxic zone during the early phase of implantation. Therefore, the semipermeable barrier and avascular properties of PDZ suggest that immunocompetent and toxic molecules are barred from entering the developing embryo. ZO-1 and E-cadherin are perhaps the contributing molecules which participate in creating this barrier.

In conclusion, E-cadherin and ZO-1 could play a central role during implantation by maintaining cell-cell linkages

FIG. 7. Immunocytochemistry of E-cadherin in mouse uterus on days 7 and 8 of pregnancy. Bright-field photomicrographs of representative uterine sections on day 7 at 0900 h (a, 40 \times and b, 100 \times) and day 8 at 0900 h (c, 40 \times and d, 100 \times) are shown. em, embryo; le, luminal epithelium; pdz, primary decidual zone.

and signaling as well as by establishing a physical barrier against immunocompetent molecules and immune cells to protect the embryo from the attack by the maternal immune system. Information about the molecular organization of adherence and tight junctions and their interactions with the other adhesive molecules is needed to better understand the roles of junctional complexes in implantation.

ACKNOWLEDGMENTS

This work was supported in part by National Cooperative Agreement on Markers of Uterine Receptivity for Blastocyst Implantation (HD29968) to S. K. Dey and by NIH Grants HD35114 to B.C.P., HD12304 to S. K. Dey, and ES 07814 to S. K. Das. Center grants in Reproductive Biology (HD-33994) and Mental Retardation and Developmental Disabilities (HD-02528) provided access to various core facilities.

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Received for publication November 5, 1998

Revised December 29, 1998

Accepted January 12, 1999